PREVALENCE OF MUTATIONS IN MDR3/ABCB4 GENE IN YOUNG PATIENTS WITH CHOLELITHIASIS

Dissertation submitted for

D.M.DEGREE EXAMINATION- AUGUST-2013

BRANCH-IV-MEDICAL GASTROENTEROLOGY

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CERTIFICATE

This is to certify that the dissertation **"PREVALENCE OF MUTATIONS IN MDR3/ABCB4 GENE IN YOUNG PATIENTS WITH CHOLELITHIASIS"**is a bonafide work of **Dr.M.RADHA** in partial fulfillment of the requirements for D.M. Branch-IV (MEDICAL GASTROENTEROLOGY) examination of THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY to be held in August 2012.The period of post-graduate study and training was from August 2010 to July 2013.

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DECLARATION

I, Dr. M.RADHA, solemnly declare that this dissertation entitled, **PREVALENCE OF MUTATIONS IN MDR3/ABCB4 GENE IN YOUNG PATIENTS WITH CHOLELITHIASIS** is a bonafide work done by me at the Department of Medical Gastroenterology, Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai during the period 2010- 2013 under the guidance and supervision of the Prof.T.Pugazhenthi M.D.,D.M., Additional Professor,Department Of Medical Gastroenterology, Professor and Head of the department of Medical Gastroenterology ofMadras Medical College & Government General Hospital, Professor Mohammed AliM.D,.D.M.

This dissertation is submitted to The Tamil Nadu Dr.M.G.R Medical University,towards requirement for the award of D.M. Degree (Branch-IV) in Medical Gastroenterology

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ACKNOWLEDGEMENTS

A great many people made this work possible. I thank my Dean for allowing me to conduct this study. I owe my warmest respects and sincere thanks to our beloved Professor T.Pugazhenthi M.D.,D.M., additional professor and Prof. Mohammed Ali Professor and Head of the Department of Medical Gastroenterology, Government General Hospital, Chennai who were the driving force behind this study. I am indebted to my Assistant Professors Dr.K.Prem Kumar, Dr.Ratnakar Kini P, and Dr. Kani Sheik Mohammed who helped in my work

I acknowledge Dr.A.K.Munirajan for his guidance and their students for supporting, and without him much of this work would not have been possible.

In addition, I am grateful to of my copostgraduate students for helping me out throughout this study period.Last but not the least I thank all my patients for their kind cooperation

CONTENTS

INTRODUCTION

Bile is secreted from the hepatocyte and it is essential for lipid metabolism, excretion of xenobiotic, and cholesterol homeostasis. Bile secretion depends upon the formation of bile acids in hepatocyte and its canalicular secretion.Once secreted in to the bile duct, bile enters the small bowel through the ampulla of water during the digestion phase. Bile stored inside the gall bladder during inter- digestive phase. After reaching the small bowel, the bile acid isabsorbed in the terminal ileum, and undergoesthe enterohepatic cycling. This happens 5-6 times per day and maintains the bile acid pool. This is essential to maintain the normal liver function.

Gall stones are formed when ever alteration in the chemical composition. Disturbance in bile acid formation,canalicular secretion and intestinal transport leads to gall stone formation.so it is important to identify hepatic, biliary, and intestinal bile acid transporters in the bile formation and secretion.

Gall stones divided into three types:

1) Cholesterol Stones,

2) Pigment Stones-(black)

3) Pigment Stones - (brown)

Cholesterol stones are more common in north India in compare with southern India where pigment and mixed stones are common. Most of the gall stones are asymptomatic in presentation.

The exact role of genetics in the formation of gallstones still not understood. The prevalence varies between the ethnic groups.

ABCB4 – encoding for the ATP-binding cassette, member of MDR3. It mediates the efflux of phospholipids from hepatocytes into canaliculi mainly the phosphatidylcholine [PC] into the bile. The function of phospholipid excretion is to protect the cell membrane from toxic bile saltsin the biliary tree.When function isdefective, it produces the spectrum of disorders, ranges from cholestasis, Cholelithiasis, Intrahepatic Cholestasis of Pregnancy, Ductopenia, and Cirrhosis.

So when ever other risk factors exclude for the formation of gall stone disease still the possibility of genetic transporters defect.

When mutation occurs at ABCB4 gene, causes the low phospholipid and high lithogenic bile.

Hence in this study we are looking for the prevalence of mutations in MDR3/ABCB4 gene in young patients with Cholelithiasis.

AIM OF THE STUDY

To study and analyze the clinical profile, association of mutations in the MDR3/ABCB4 in young patients age less than 40 years with Cholelithiasis

REVIEW OF LITERATURE

Liver is the largest organ of the body compressing of one fifth of total weight about 1200-1500 gms. Liver has dual blood supply receives 70% of blood supply from portal vein, 30 % receives from hepatic artery. Has two lobes and eight segments depends upon the distribution of blood supply.

Histology of liver

The hepatocytes arranged in plates with one to two cell thicknesses. They are separated by the sinusoids. Portal tract contains the branches of portal vein, hepatic artery, and bile duct.

The bile formed in the hepatocyte is excreted through the canaliculi. These canaliculi have no wall and lined by tight junction of hepatocytes. This drain into intra lobular canalicular network, canals of Hering then into interlobular bile duct, small, medium, large bile duct .The right and left hepatic duct unite and forms the common hepatic duct, it joins with cystic duct and forms the common bile duct.

The bile ducts are lined by cholangiocytes. The functions of cholangiocytes include modification of bile, secretion of water and

electrolytes, absorption of the bile acids and plays major role in chole - hepatic shunt pathway.

Bile is a complex solution, osmolality similar with plasma, composed with water, electrolytes, bile acids, phospholipids bile pigments, and cholesterol.

About 500-600 ml of bile secreted each day. A major constituent is bile acid. It is actively secreted into the canaliculus. When bile acid secreted, it is actively induces the secretionof other solutes like phospholipids and cholesterol .Bile flows down into the gall bladder and stores there. When meal reaches the duodenum ,it is stimulatesthe release of cholecystokinin which cause the contractionof gall bladder and release of stored bile[1]and facilitates the absorption of cholesterol and reaches the ileum and absorbed through the ileal transporters and returned back to the portal circulation and enters into the liver[1].

Functions of bile acids

1. Digestion of lipids, cholesterol metabolism and fat soluble vitamins

2. Maintains the antimicrobial defense in the gut by forming the fat micellar complex[2].

3. Itprevents formation of calcium gall stonesand oxalate kidney stones[3]

4. Regulate the entero hepatic circulation

Bile synthesis

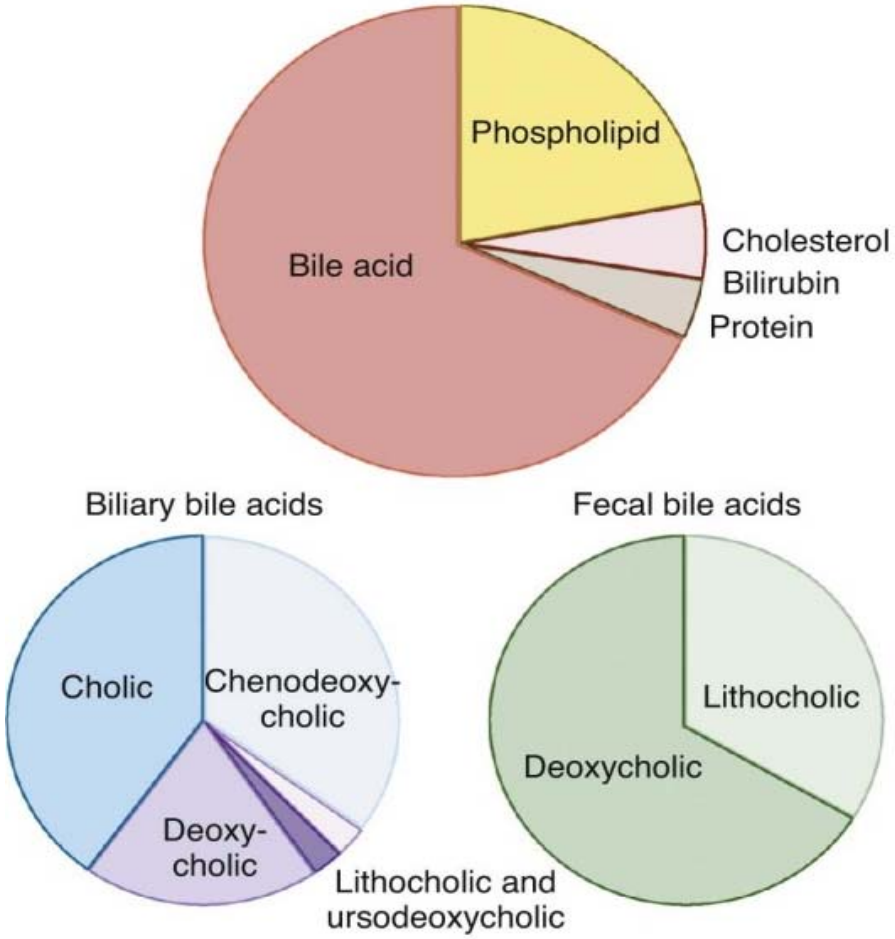
Bile acids synthesized from cholesterol from two pathways, the classic pathway and the alternate pathway. The primary bile acids composed of cholic acid and chenodeoxy cholic acids, which conjugate with glycine andtaurine, produce the secondary bile acids, to enhance the soluble nature of the bile acids.Endogenous bacterial flora present in the colon deconjugates the bile and the bile acids are absorbed in to the circulation.

Composition of Hepatic Bile[6]

COMPONENT	**CONCENTRATION**
Electrolytes and minerals (mmol/L):	
Sodium	140-160
Potassium	3-8
Chloride	70-120
Bicarbonate	20-50

COMPONENT	CONCENTRATION
Calcium	1-5
Phosphate	0-1.2
Magnesium	1-3
Metals (?mmol/L):	
Iron	2-72
Copper	12-21
Organic constituents (mmol/L):	
Bile acids	5-50
Bilirubin (total)	1-2
Phospholipid (lecithin)	0.5-20.0
Cholesterol	0.5-1.0
Glutathione	3-5
Glucose	0.2-1.0
Urea	2.2-6.5
Protein (g/ld.)	0.2-3.0

Composition of bile acids



HEPATIC BILEACID TRANSPORT

The Hepatocyte and the Cholangiocyte areinvolved in the formation of bile. The hepatocellular movement of bile occurs via following three mechanisms.

1. Primary ATP dependent,

2. Secondary Sodium Gradient dependent,

3. Tertiary OH-or HCO3 dependant.[4]

Active secretion of organic and inorganic solutes followed by passive movement of water and electrolytes occurs during production of bile.

There are two **types of bile flow** into canaliculi

1. Bile acid dependent bile flow

2. Bile acid independent bile flow

Primary solutes-actively pumped are conjugated bile acids, conjugated bilirubin, glutathione, and heavy metals.

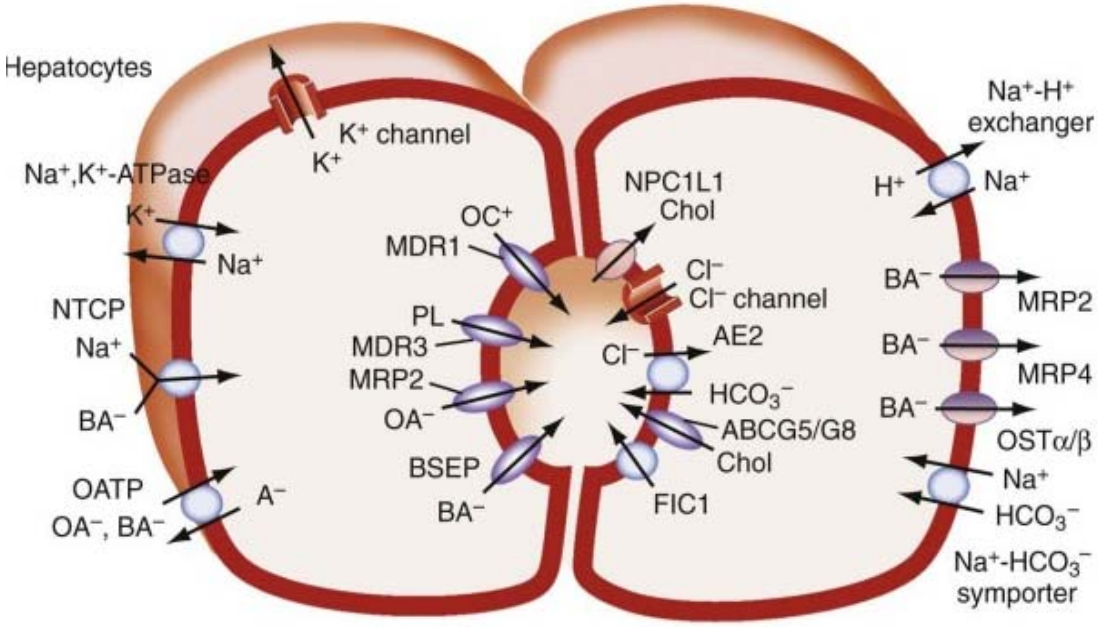
Secondary solute-water plasma, electrolytes,glucose, and amino acids

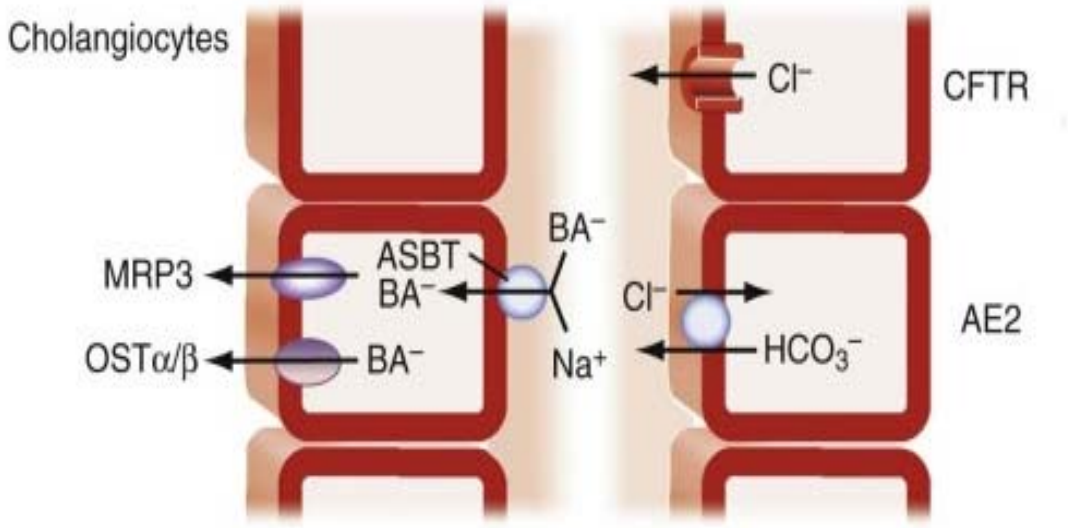
Chole-hapatic shunt pathway The unconjugated bile acids secreted into the bile absorbs into the bile duct epithelial cells and periductular plexus and resecreted into the bile. This absorption generates a bicarbonate anion leads to bicarbonate rich bile acid secretion[5].

BILE ACID TRANSPORT PROTEINS

They are present in human hepatocyte,cholangiocyte,ileal enterocyte,and renal proximal tubule.The hepatocyte and sinusoidal membrane expressthe specialized transport protein for various compounds.

HEPATIC AND CHOLANGIOCYTE TRANSPORTERS





BILE ACID SECRETION DEPENDENT TRANSPORTERS.

Transporter	Site	Function
NTCP	Basolateral	Na + dependent bile acid/xenobiotic uptake
OATP	Basolateral	Na + in dependent bile acid/xenobiotic uptake
Na +k+ ATPase	Basolateral	Secretion of 2 Na for 3 K+
BSEP	CANALICULAR	ATP dependent bile acid transport
MDR3	CANALICULAR	ATP dependentphosphatidylcholine transport
ABCG8	CANALICULAR	ATP DEPENDENT STEROL EXPORT
FIC1	Canalicular	ATP dependentamino phospholipid flipping

BILE ACID INDEPENDENT BILE FLOW-TRANSPORT PROTEINS

MRP 2	Canalicular	ATP dependent GLUCURONIDE,GLUTATHIONE,SULFATE CONJUGAES
OATP	BASOLATERAL	NA+INDEPENDENT ORGANICANIONS,CATIONS, NEUTRAL STEROIS

SINUSOIDAL BILE ACID EXPORT

MRP 3	Basolateral	ATP dependent export of bile acids and glucuronide conjugates
MRP4	BASOLATERAL	ATP dependent export of glutathione & bile acids
OSTα-OSTβ	BASOLATERAL	Bile acid export

CHOLANGIOCYTE/DUCTULAR SECRETION

Aquaporin 1 (AQP1)	Apical membrane	Water transport
Aquaporin 4 (AQP4)	Basolateral membrane	Water transport
AE2 (SLC4A2)	Apical membrane	HCO_3^- secretion in exchange for Cl^-
CFTR (ABCC7)	Apical membrane	Cl^- secretion
ASBT (SLC10A2)	Apical membrane	Bile acid uptake (Chole-hapatic shunt

ILEAL ENTEROCYTE

ASBT (SLC10A2)	Apical membrane	Na^+-dependent bile acid uptake
NPC1L1	Apical membrane	Sterol import
OSTα-OSTβ	Basolateral membrane	Bile acid export
MRP3 (ABCC3)	Basolateral membrane	Bile acid export

80 %bile acids uptake is Na^+ dependent and it is against concentration gradient. The driving force generated by NA^+K^+ATPase. It maintains the Na^+ ion gradient mainly through the NTCP,which also responsible for the uptake of major drug like Rosuvastatin. Any

polymorphism in this gene is present asasymptomatic,because liver also express Na+ independent bile acid transporters.

Rate limiting step in bile acid transport is canalicular secretion.In canaliculi, the bile acid concentration is more than 1000 fold as compared to concentration inthe hepatocytes. Bile acid secretion into the canaliculi is mediated through the MDR1 –multi drug resistance protein 1renames as Bile Salt Export Pump. Mutation in these results in progressive familial intra hepatic cholestasis type 2 characterized by biliary bile acid concentration <1%.

INBORN ERRORS OF BILE ACID SYNTHESIS AND TRANSPORT[7]

Defective Bile Acid Synthesis	
Primary defects	Cerebrotendinous xanthomatosis (C_{27}-steroid-27-hydroxylase deficiency)
	Δ^4-3-oxosteroid 5β-reductase (AKR1D1) deficiency
	C_{24}-steroid-7α-hydroxylase (CYP7B1) deficiency
	α-Methylacyl-CoA racemase (AMACR) deficiency
Secondary defects (due to organelle damage)	Peroxisomal biogenesis disorders (PBDs)
	Rhizomelic chondrodysplasia punctata
	Zellweger spectrum

Zellweger's syndrome

Neonatal adrenoleukodystrophy

Infantile Refsum's disease

Other

Hyperpipecolic acidemia

Leber's congenital amaurosis

Disorders with loss of single peroxisomal function

Acatalasemia

Acyl-CoA oxidase deficiency

Adult Refsum's disease

D-bifunctional protein deficiency

Hyperoxaluria type I

Sterol carrier protein X deficiency

Thiolase deficiency (pseudo-Zellweger's syndrome)

X-linked adrenoleukodystrophy

Contiguous gene syndrome

Generalized hepatic synthetic dysfunction

Fulminant hepatic failure (multiple causes)

Neonatal iron storage disease

Tyrosinemia

Defective Bile Acid or Phospholipid Transport
FIC1 (FIC1) deficiency: progressive familial intrahepatic cholestasis (PFIC) type 1:
Byler's disease
Benign recurrent intrahepatic cholestasis (BRIC) syndrome
Greenland familial cholestasis
BSEP (ABCB11) deficiency: PFIC type 2
MDR3 (ABCB4) deficiency: PFIC type 3
Others
Alagille (Jagged) syndrome
Cholestasis-lymphedema syndrome (Aagenaes syndrome)

BILE ACID TRANSPORT DEFECTS

There are various spectrum of disease are associated with mutation of these defects. Three important diseases in this spectrum are, familial intrahepatic cholestasis (FIC1) disease, bile salt export pump (BESP) disease, multi drug resistance protein 3 (MDR3) diseases.

Familial intrahepatic cholestasis (FIC1) disease

This disease is caused by mutations in the **ATP8B1**gene; lead to progressive early-onset FIC1 disease (PFIC type 1).It typically shows a bland canalicular cholestasis, with varying degrees of hepatocellular ballooning and giant cell transformation; portal fibrosis and eventually cirrhosis.

BSEP disease

This disease is caused by a wide spectrum of mutations in the **ABCB11** gene. Patients will presents with high serum bile acid levels, but low or low-normal serum GGTP levels, nonspecific giant cell hepatitis on routine histology, increased risk of developing malignancies of the hepatobiliary system, such as hepatoblastoma, hepatocellular carcinoma, and cholangiocarcinoma, in contrast to patients with other forms of progressive intrahepatic cholestasis.

MDR3 disease

MDR3 deficiency is leads to decreased excretion of cytoprotective biliary phospholipids and causes leaving an increased pool of cytotoxic biliary bile salts. This can leads subsequent bile duct damage and proliferation.

This disease caused by mutations in the ABCB4 gene that encodes the MDR3 glycoprotein. This helps translocate phospholipids into canalicular membrane.Present with several disease like PFIC type 3, intrahepatic and gallbladder lithiasis, intrahepatic cholestasis of pregnancy, and adult-onset ductopenic cholestatic liver disease[8].

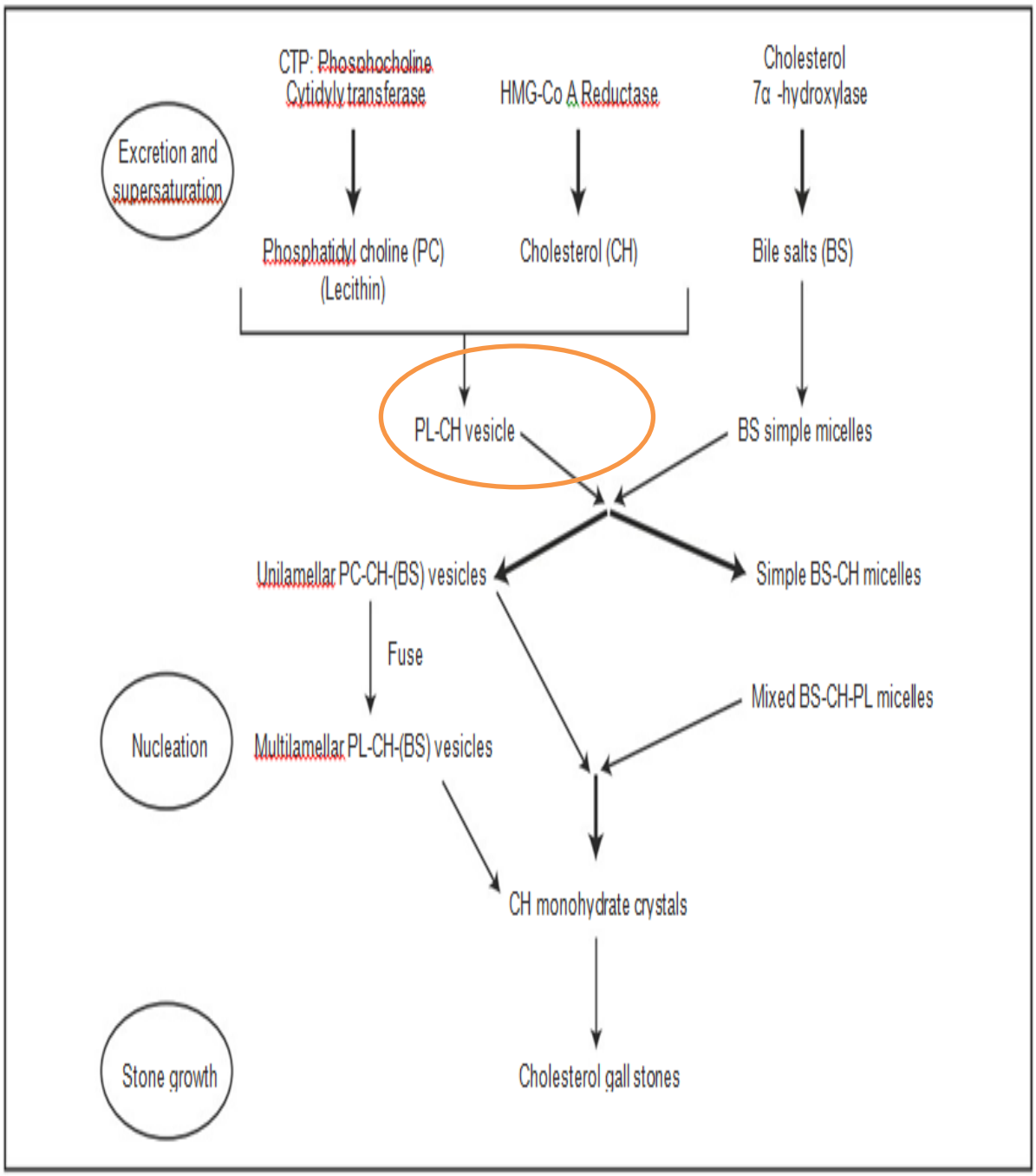
This mutations act as modifiers in disease like primary sclerosing cholangitis, primary biliary cirrhosis, and drug-induced cholestasis.

PFIC -3 has high levels of serum GGTP and bile acids, and bile ductular proliferation on routine microscopy.

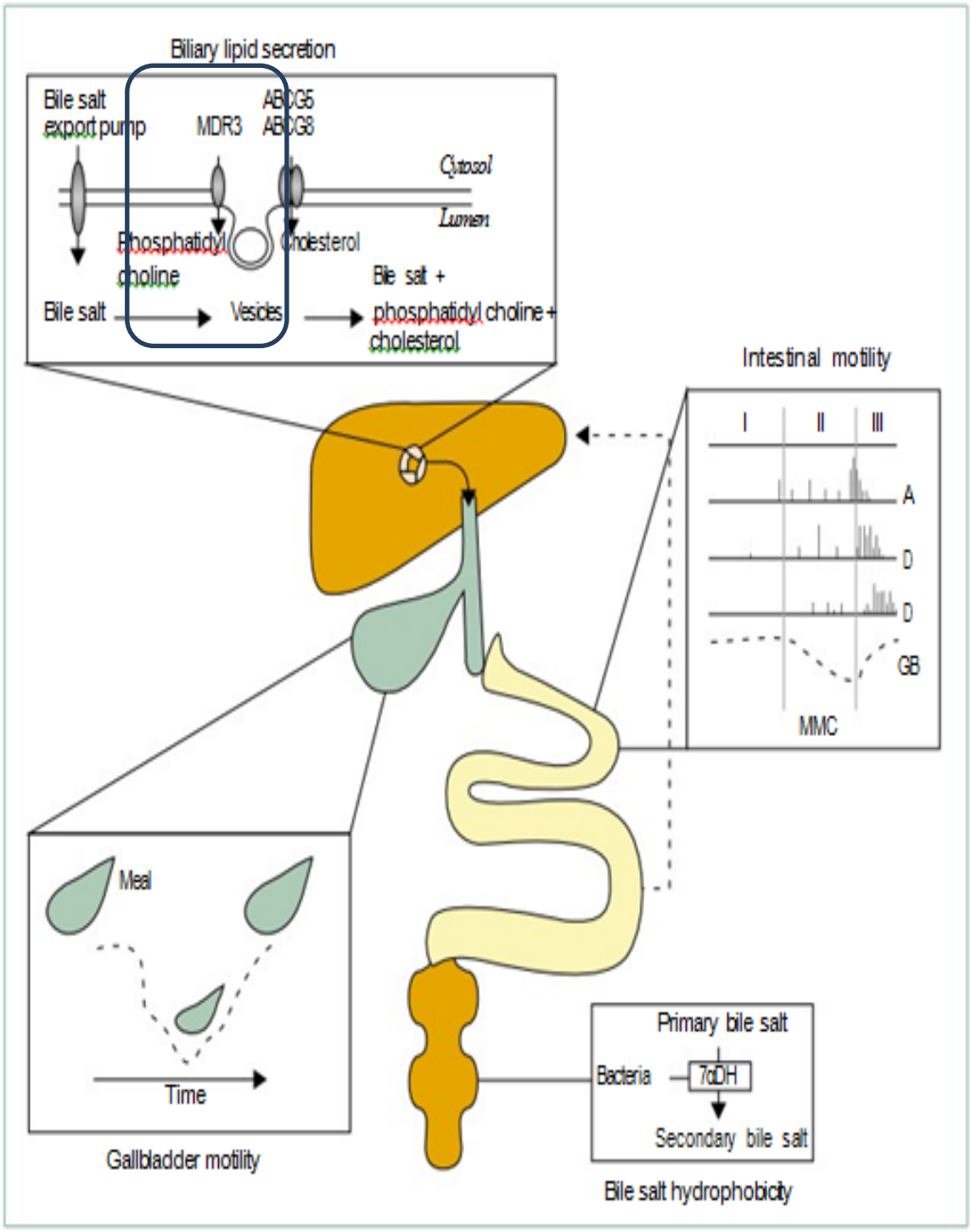
In female patients with familial intrahepatic cholestasis of pregnancy showed heterozygous mutation in this gene.

The mutations likely lead to a genetic predisposition that requires the coexistence of other nongenetic factors for full expression of the disease

Pathogenesis of gall stones [10]



The Role of Liver, Gallbladder And Intestine In Cholelithiasis[9]



Risk factors for gall stones[10]

Modifiable	**Non modifiable**
Obesity • Rapid weight loss • Diet, • Alcohol abstinence • Decreased physical activity • Smoking • Medications (octreotide, ceftriaxone) • Hyperlipidemia • Diabetes mellitus type	Female Gender • Age • Genetics

In small percentage of people where all other risk factors are eliminated, the genetics plays a major role in the formation of gall stones. The biliary secretion of lipids appear important initial step the formation of gall stones.so when these defects are present they are the high risk patients to recurrent symptoms and it is important to identify these subgroup of patients and surveillance for the further developments of disease spectrum

The genetic involvement in this group of patients varies with different ethnicity[10].The highest prevalence seen in prima tribe of Arizona 73 % of women >25 years have gall stones with prevalence raises 90 % in>60 years[11]. In Europe highest in Norway -21%, lowest in Italy-6%

In northern India- 6% with the increased consumption of Western diet increase of this prevalence can occur[12].

Functional characters of ABCB4 gene[14]

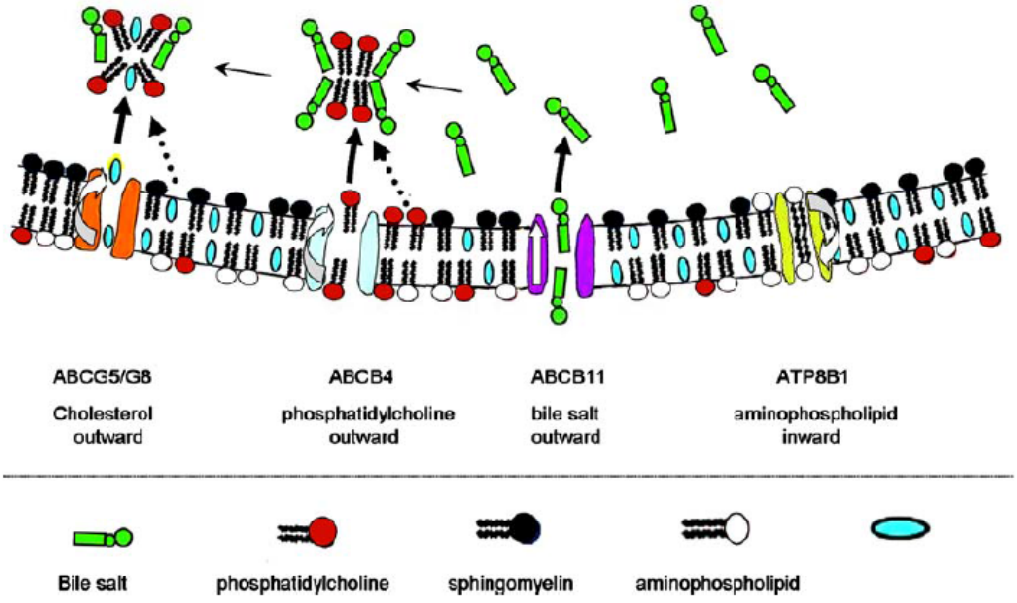
The gene studied in mice that ,the wild type mice do excrete considerable amount of phospholipid as compared the mice with disrupted gene do not excrete phospholipid [13].ABCB4 is predominantly express in the liver small amount excreted in adrenal gland, muscle, testis, eye and placenta.

De Vree et al showed that here is a generalized bile acid reduction when function of ABCB4 decreased [16].

The canalicular membrane has to tolerate very high concentratedetergent actions of bile salts well above the critical micellar concentration. Thiscan leads to solubilization of membranes[17]. Protective effect mediates through the transport of phospholipid through the canalicular membrane by via ABCB4 leads to reduction in concentrated bile acids and reduces the amount of injury.

When the patients treated with UDCA, the ABCB4 gene mRNA levels are not increased, but the level of ABCB4 protein level is increased by post translational modification[15].

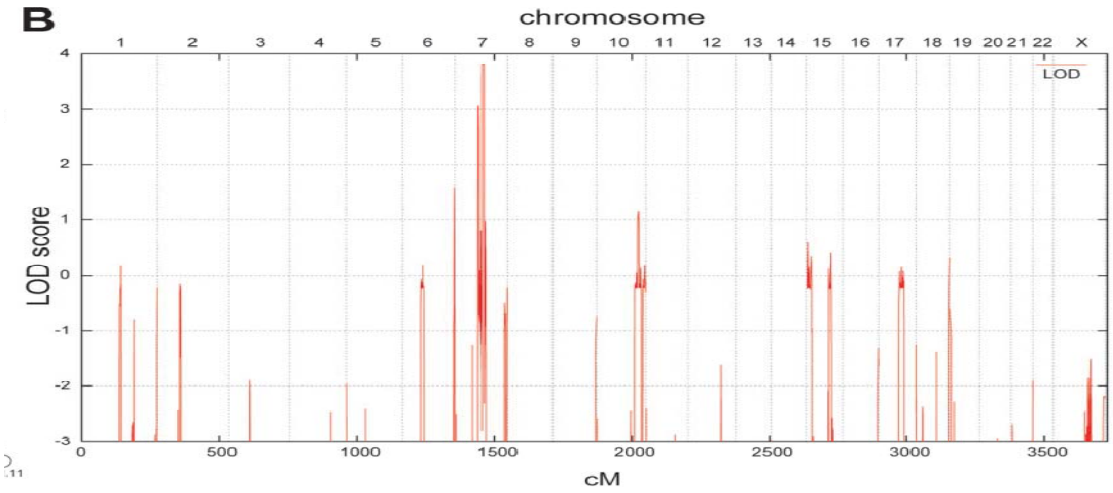
Hypothetical lipid excretion at canaliculi[14]



Canalicular lipid transport defects can cause gallstone formation. Because the solubilization the amount of bile salt and phospholipids [18]. In ABCB negative mice the absence of phospholipid excretion leads to development of gall stones [19]. So in patients with ABCB4 mutations are more prone to develop gall stone formations.

Predictors of mutations in ABCB4 in patients with Cholelithiasis are symptoms reoccur after surgery, age <40 years, intra hepatic hyper echoic material[20].

When analyzing the mutations present in ABCB4 gene, GOTTHARDT, RUNZ, ET AL [8] demonstrated in familial cases of cholestasis the highest lod score region of chromosome number 7.



Olivier Rosmorduc et al[21] done ABCB4 gene sequencing, of exons 2 to 28 and all splice junctions in patients with Low phospholipid associated Cholelithiasis and found identified 14 heterozygous and homozygous pointMutations .

LANG ET AL[22] identified several polymorphism in the ABCB4 gene, done in various ethnicgroups consists of Caucasians, Korea, andJapanic populationsin both exon and intron of ABCB4 gene.

Dario DE GiorgioET a[231] identified 25 new mutations in the ABCB4 gene in patients presented with progressive intrahepatic cholestasis, showed a cluster in the exon 17. As in our study we decided to study the exon 17 of ABCB4 gene in youngpatients less than 40 years presented with Cholelithiasis.

MATERIALS AND METHODS

STUDY CENTER

Department of Medical Gastroenterology Madras Medical College & Rajiv Gandhi Government General Hospital, Chennai,and Institute of Basic Medical Sciences,Madras University, Taramani,Chennai

DURATION OF STUDY

From April-2012 to Feb -2013

SAMPLE SIZE

20

INCLUSION CRITERIA

Patients meeting the following

1. Age less than 40 years
2. Cholelithiasis, choledocholithiasis
3. Cholangitis, calculus cholecystitis, acute pancreatitis
4. Intra hepatic biliary sludge
5. Family history of gall stones
6. Post cholecystectomy pain syndrome
7. Intra hepatic cholestasis of pregnancy

EXCLUSION CRITERIA

1. Age more than 40 years
2. Hemolytic disorder
3. Auto immune disorder
4. Wilsons disease
5. Obesity
6. Metabolic Syndrome
7. NAFLD
8. Alcoholic liver disease

SAMPLE COLLECTION:

5 ml of peripheral blood was obtained by direct vein puncture using disposable syringe from each subject. The samples were immediately transferred into EDTA containing centrifuging tubes. The tubes were kept in ice box and transferred immediately to the laboratory. The buffy coat was separated and stored at –20°C until isolation of DNA was done. Blood samples recruited for the study was collected from young patients with Cholelithiasis referred to Department Of Medical Gastroenterology, Madras Medical College, and Chennai.

Prior ethical permission was obtained from the ethical committee and blood sample was collected from each patient after a well-informed structure of the study. The relevant clinical details was also collected and

tabulated and the samples were grouped based on their clinical status. The isolated from the blood samples sentto analysis for the presence of Single Nucleotide Polymorphisms (SNP) in the ABCB4 gene.

DNA ISOLATION AND PURIFICATION:

The phenol chloroform method of DNA isolation was used in this study. This frequently used method for DNA isolation removes proteins and other cellular components from nucleic acids, resulting in relatively pure DNA preparations.

Principle:

The concept of isolation of DNA is that all the other components of the cell and chromatin are removed using suitable methods to leave behind the DNA. In general the isolation of DNA from mammalian tissues follows four different steps.

1. Lysis of cells with a detergent like sodium dodecyl sulphate (SDS).
2. Digestion of proteins with enzymes (Proteinase – K).
3. Extraction of DNA by phenol chloroform method.
4. Precipitation of DNA with 100% ethanol.

Reagents and their Functions:

1. RBC Lysis Buffer

Ammonium chloride – 155mM (8.29g)

EDTA – 0.1mM (1.00g)

$NaHCO_3$ – 12mM (0.034g)

Adjust pH to 7.4 with 1M HCl or NaOH; make up to 1000ml with distilled water. Autoclave and store at room temperature. The RBC Lysis buffer is used to lyse the erythrocytes.

2. SE Buffer/WBC Lysis Buffer

Na2EDTA – 25mM (8.41g)

NaCl – 200mM (11.69g)

Adjust pH to 8.0 with 1M NaOH; make up to 1000ml with distilled water, autoclave and store at room temperature.

3. Proteinase K (10 mg/ml)

Dissolve 100mg Proteinase K in 10ml distilled water at room temperature and store it at -20°C. Proteinase K is the enzyme commonly employed for digestion of proteins. It is a highly active protease purified from the mold Tritirachium album.

3. Sodium dodecyl sulphate (SDS) 10%

SDS - 10 gram

With 10gram of SDS add distilled water to make up to 100 ml, stir on a magnetic stirrer, filter and store at room temperature. Do not autoclave. SDS is the commonly used detergent for DNA isolation. It ruptures the cell wall and nuclear membranes to release the contents. Furthermore, it also denatures proteins present in the sample.

4. **Phenol (Saturated, pH 8)**

Phenol is used to extract the DNA from the solution. In alkaline pH it extracts the DNA to the aqueous phase, which is collected for further purification. This will prevent the contamination of DNA with RNAs. In neutral or acidic pH phenol extracts RNA to aqueous phase. Hence, the pH of phenol is very important for this step. The pH of phenol should be maintained above 7.8 as all eukaryotic RNA with poly-A tails dissolve in alkaline phenol but in the acid range the DNA will partition into organic phase.

5. Phenol: Chloroform: Isoamyl alcohol mixture

To prepare Phenol: Chloroform: Isoamyl alcohol mixture, mix 25 parts of Phenol, 24 parts of Chloroform and 1 part of Isoamyl alcohol.

The denaturation of proteins is mainly achieved through the activity of chloroform. It causes surface denaturation of proteins and also helps in removal of fats from the sample. Chloroform also eliminates any traces of phenol. Because phenol cause phosphodiester breakage, andalso useful for the removal of protein from nucleic acid samples.

Due to the presence of proteins cause more chance of foaming in the solution at the time of phenol: chloroform extraction.This can be reduced by addition ofIsoamylalcohol and to maintain the stability of layers after centrifugation of deproteinised solution.

6. Absolute ethanol

The action of 100% ethanol is to precipitate the DNA leaving debris RNA and polysaccharides in the solution.

7. 70% Ethanol

Ethanol	-	70 ml
Distilled water	-	30 ml

It removes residual salt and moisture in the precipitated DNA.

8. Tris-EDTA Buffer (pH - 8.0)

Tris base	-	1.2114 gram
EDTA	-	0.0372 gram

This dissolved in 900 ml distilled water. Adjust the pH to 8. made up the volume to 1000 ml. Filter it , autoclave & store at 4^{o}C. This is an ideal buffer to store the DNA.

Procedure:

1. 5ml of whole blood was taken and spun at 3500rpm for 20min at 25°C.

2. Buffy coat was removed carefully and transferred to a new 2.0 ml eppendorf tube.

3. 1ml of 1X RBC lysis buffer was added to the buffy coat, vortexed, mixed well. Incubated for 15 min at 37°C, followed by a spin at 3500 rpm for 15 min at room temperature.

4. The supernatant was discarded, the pellet was dislodged and washed 2 or 3 times with 1ml RBC lysis buffer (repeat of step 3) until a half white pellet appears. 5. The pellet was then dislodged by tapping to which 500µl of SE (WBC lysis) buffer, 5µl Proteinase K (final concentration 50µg/ml) and 25µl of 10% SDS (final concentration 0.5%) was added and incubated in water bath at 37°C for overnight or 55°C for 3 hours.

6. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) to the lysate was added and intensely mixed well by inverting the tube until it turns to milky white in colour.

7. The samples were spun at 10,000 rpm for 15min at room temperature.

8. The upper aqueous phase alone was carefully collected with the help of wide bore tips without disturbing the other layers and transferred to a new tube.

9. 2.5 volume of cold absolute ethanol was added to this aqueous phase and the tubes were inverted gently for several times. The DNA was found to be visible like a thread and assumed the shape of a cotton ball.

10. The DNA was transferred to an eppendorf tube already containing 1ml of 70% ethanol and spun at 12,000g for 10 min at 4°C.

11. Supernatant was discarded and the pellet was air-dried in a sterile place for 3 hours to remove any trace of residual ethanol.

12. Appropriate amount of 1X TE was added according to the size of the pellet, allowed to dissolve and stored at 4°C.

QUALITY CHECK AND QUANTIFICATION OF DNA

The integrity of the DNA was assessed by running it on 0.7% Agarose gel. Further the quantification and quality check of DNA was performed by subjecting the DNA to spectrophotometry. The concept of quality check of DNA is to find out the purity of the extracted DNA. The extracted DNA may contain impurities like phenol, proteins and others.

Principle for Agarose gel electrophoresis

The integrity of the DNA is checked by Agarose gel electrophoresis. When the DNA is mixed with loading dye and run electrophoretically on 0.7% Agarose gel in TAE buffer, the good high molecular weight DNA will appear as sharp band without smearing.

Reagents

1. TAE buffer (10x)

Tris base	-	48.4 gram
Glacial acetic acid	-	11.42 ml
0.5 M EDTA (pH 8.0)	-	20 ml

Distilled water to make up to 1000 ml. Autoclave and store at room temperature.

2. Gel loading dye – Type III (6x)

Bromophenol blue	-	0.25% (w/v)
Xylene cyanol FF	-	0.25% (w/v)
Glycerol in water	-	30% (v/v)

Stir well in and store at 4oC.

3. Ethidium bromide

Ethidium bromide	-	10 mg
Distilled water	-	1 ml

Mix well to ensure that the dye has dissolved completely. Wrap the tube in aluminum foil and store at room temperature.

Procedure

1. 0.7% Agarose Gel preparation

i. 0.7 gram of Agarose was weighed and transferred into a 250 ml conical flask.

ii. 100 ml of 0.5x TAE buffer was added to it, stirred well and melted on a magnetic stirrer cum hot plate until the Agarose dissolves completely.

iii. The appropriate sized gel tray and comb was washed and wiped with 70% Ethanol. The gel tray was placed inside the casting unit. The comb was placed on the gel tray and left on an even surface.

iv. After the Agarose cools down to hand bearing temperature, 5µl of ethidium bromide was added and mixed well. It was poured on the gel tray and allowed to polymerize. After polymerization the comb is removed gently.

2. Preparation of sample and loading

i. The gel tray was removed from the casting unit and the tray placed in the electrophoresis tank.

ii. 0.5x TAE buffer was poured into the tank until the gel gets immersed.

iii. 2µl of each DNA sample was taken and mixed with 2µl of 6x loading dye and 8ml of sterile double distilled water.

iv. The DNA samples were loaded into the wells.

v. The electrodes were connected.

vi. The power was switched ON, set at 100 V.

vii. As the DNA is negatively charged, it will migrate towards the anode.

3. **Visualizing the DNA**

i. When the bromophenol blue dye was in the middle of the gel, the power was switched OFF.

ii. The gel was taken to the transilluminator and observed under UV and documented.

iii. The good high molecular weight DNA will appear as sharp band without smearing.

Procedure for spectrophotometry

The nucleic acid sample was analysed at 260nm and 280nm by using Nanodrop Spectrophotometer (Thermo scientific, Germany). The concentration and purity of the sample was analysed using the following formula,

Concentration of DNA

Concentration of double stranded DNA sample (μg/μl) = A260 x 50

Purity of DNA

Pure DNA = A260 /A280 ≥ 1.8

< 1.8 indicates protein and phenol contamination.

> 2.0 indicates the possible contamination with RNA.

DNA DILUTION

After confirmation for the presence of genomic DNA in the sample and quantification, the sample has to be diluted with autoclaved sterile double distilled water, to make it amenable to be used in polymerase chain reaction. The amount of DNA needed for PCR is 50-100 ng of DNA for a 20 µl reaction mixture.

Once a working DNA sample has been prepared, it was run in 1% agarose gel electrophoresis, observed under UV and documented.

POLYMERASE CHAIN REACTION

Principle

PCR entails enzymatic amplification of specific DNA sequences using two oligonucleotide primers that flank the DNA segment to be amplified. n of large quantities of a specific DNA sequence took a leap forward with the development of the PCR. The PCR requires two nucleotide oligomers (Primers) that hybridize to the complimentary DNA strands in a region of interest. The method relies on thermal cycling, - of cycles of repeated heating and cooling done for DNA melting & enzymatic replication of the DNA.

Reagents

1. 10x PCR buffer (Applied Bio systemsInc. - ABI., USA)

2. 25 mM $MgCl_2$ (ABI., USA)

3. 2.5mM dNTP Mix (Takara, Japan)

4. Primers (Sigma Aldrich, India)

Preparation of the primer

The primer is obtained as lyophilized powder and is reconstituted in appropriate volume of sterile triple glass distilled water to a concentration of 100µM. A working stock of 2µM primer is prepared and stored at -20°C.

The primers used in this study are as follows:

Primer Name	Sequence (5'→3')	Length(bp)	Tm (°C)	GC (%)
ABCB4 ex17 F	GAGGCCAGAATAGGGACGGGC	21	60	67
ABCB4 ex17 R	GAGGTTGGGAGAAGCAGCAGC	21	58	62

Taq DNA polymerase(Applied Biosystems, USA)

It is a highly thermo stable DNA polymerase of a thermophilic bacterium Thermus aquaticus.

Procedure

i. 2µl of genomic DNA (100ng/µl) was pipetted directly to the bottom of the labeled PCR tubes.

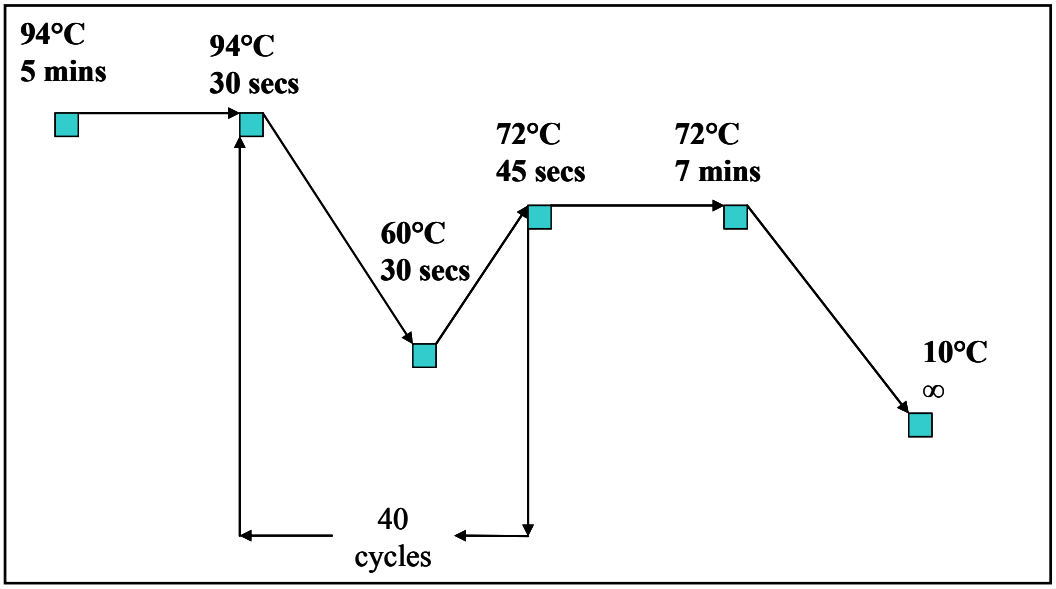
ii. A master mix of all the components of PCR except the genomic DNA was prepared as follows:

CONTENTS	STOCK CONCENTRATION	FINAL CONCENTRATION (50µl)	QUANTITY
PCR Buffer	10X	1X	5.0µl
$MgCl_2$	25mM	2.5mM	5.0µl
dNTP mix	2.5mM	100µM	2.0µl
Forward Primer	2µM	80nM	2.0µl
Reverse Primer	2µM	80nM	2.0µl
Taq DNA Pol	5U/µl	1U/Rxn	0.2µl
Distilled water	-	-	31.8µl
Genomic DNA	100ng/µl	200ng/10µl	2.0µl
Total			**50.0 µl**

Thermal cycle protocol

The PCR mix was added into a sterile 0.2ml PCR tube and the following thermal cycle condition was programmed in GeneAMP® PCR System 9700-Applied Biosystems, USA.

PCR thermal cycle



After completion of the programme, the samples were tested for amplification by 1.5% agarose gel electrophoresis. We can visualize with ethidium bromide.

The polymerase chain reaction product yields an amplicon of size 475 bp

GENOTYPING BY DIDEOXY SEQUENCING

Sequencing is a quick and flexible genotyping technique. This strategy is used to identify and confirm the presence of heterozygous single nucleotide polymorphisms within a genomic region of interest. The PCR products were sequenced commercially. (Macrogen Inc. Seoul) The protocol followed by them is as follows

Reagents

- Agarose gel (1%)
- BigDye Dilution Buffer (2.5x, Applied Biosystems)
- BigDye Terminator v3.0 (Applied Biosystems)
- dNTPs (10mM each)
- Ethanol (80%)
- Formamide (Optional)
- Forward primer (2mM)
- Genomic template DNA
- PCR Buffer (10x, from supplier of Taq polymerase)
- Precipitation mix – For 1 liter, mix 800ml of 96% ethanol, 16ml of Na Acetate (pH 5.5) and 158ml of MilliQ H_2O
- Reverse primer (2mM0

- Sequencing primer (2mM; one of the oligonucleotides used for PCR amplification can be used)
- Taq polymerase (5U/μL)

Equipment

- Apparatus for agarose gel electrophoresis
- Capillary sequncer (Applied Biosystems 3730)
- Plates are used to perform reactions
- Sequence analysis software (Bio Edit)

Method

1. The PCR product was purified using a kit method.
2. The following dideoxy sequencing rection (5μL total volume) was prepared:
    - 1μL of diluted PCR product
    - 1μL of sequencing primer (2mM)
    - 0.2μL of BigDye terminator v 3.0
    - 1.8μL of 2.5x BigDye Dilution Buffer
    - 1μL of MilliQ H_2O.
3. The following sequencing protocol was followed:

40 cycles of 92°C for 10 seconds followed by 50°C for 5 seconds and 60°C for 120 seconds.

4. Purified by adding 30µL of precipitation mix.

5. They were then vortexed for 15 seconds.

6. They were then centrifuged at 3500g for 40 minutes.

7. The supernatant was discarded (centrifuge plate upside down for 1 minute at 32g).

8. The pellet was washed by adding 25µL of 80% ethanol.

9. They were then centrifuged at 3500g for 5 minutes.

10. The supernatant was discarded (centrifuge plate upside down for 1 minute at 32g).

11. The pellet was air-dried or heated for 10-15 minutes at 80°C (until there is no smell of alcohol).

12. The pellet was dissolved in 10µL of H_2O or Formamide.

13. The samples were analyzed on a capillary sequencer (Applied Biosystems 3730).

14. The sequencing data was then analyzed using a suitable software package.

RESULTS

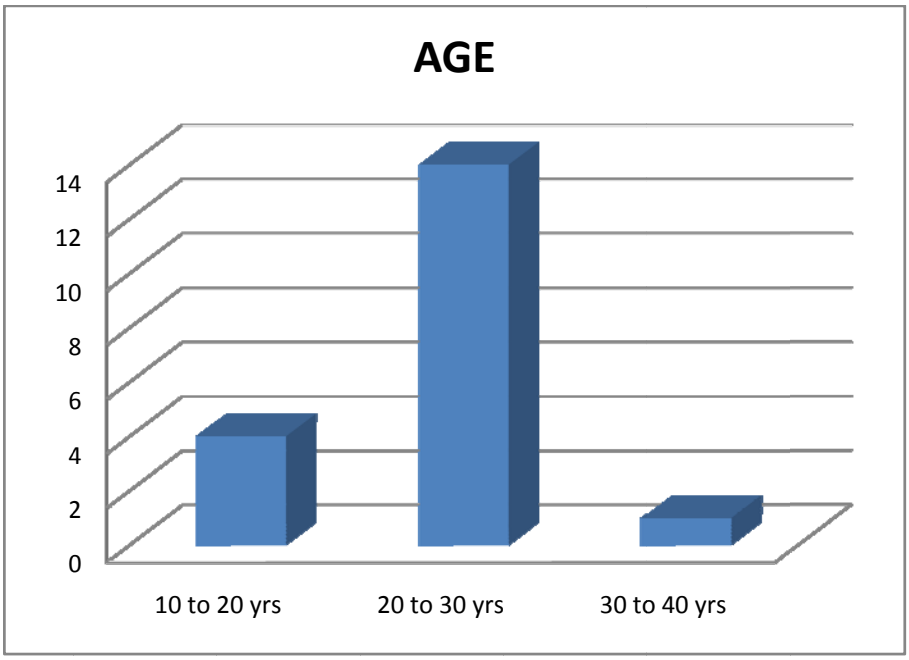
Total 20 patients consist of 3 males and 17 females were included in the study. DNA isolation done 19 patientscould notbe isolated in one patient. Finally samples from 19 patients sent for sequencing analysis.

Basic characters

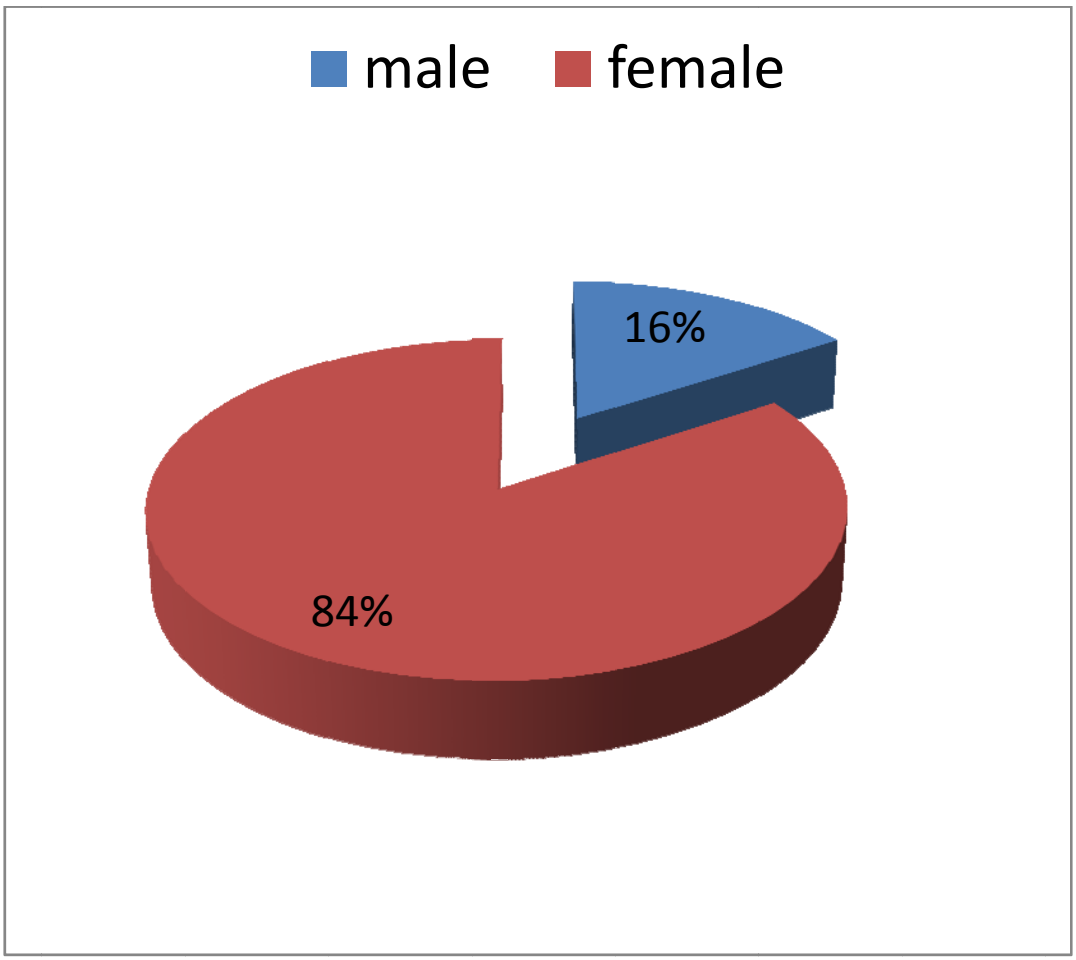
Age distribution

Range- 17-31 years

Mean age-23.8 years



SEX DISTRIBUTION



STEP NO: 1

Genomic DNA Isolation from 19 patients in 0.7% Agarose gel from the patient's blood placed in the test tube.

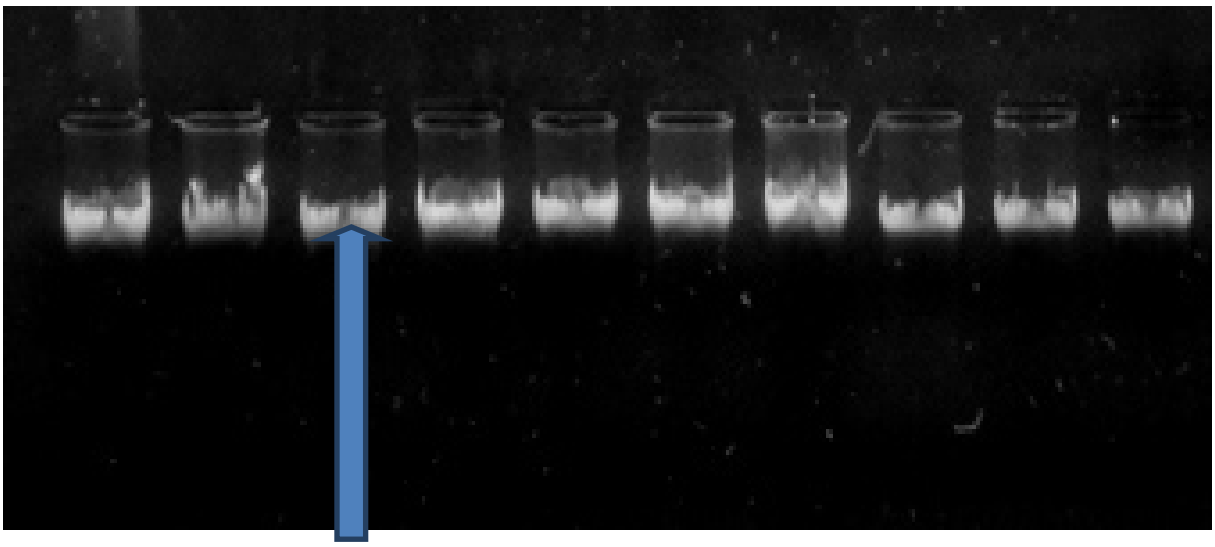
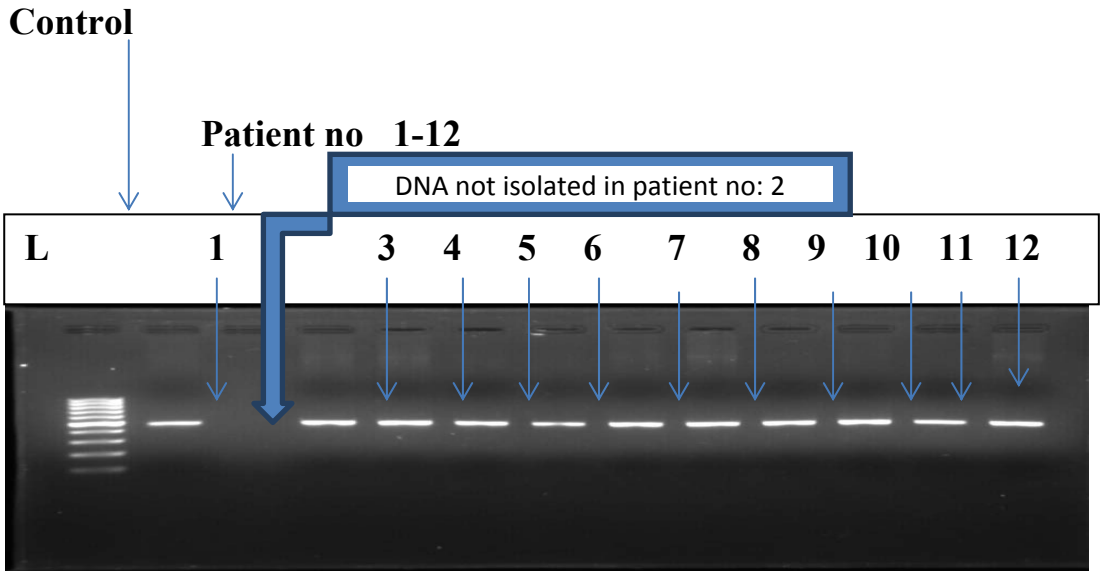


Fig No: 1GENOMIC DNA

STEP NO: 2 PCR amplicon of ABCB4 gene by electro phoresis



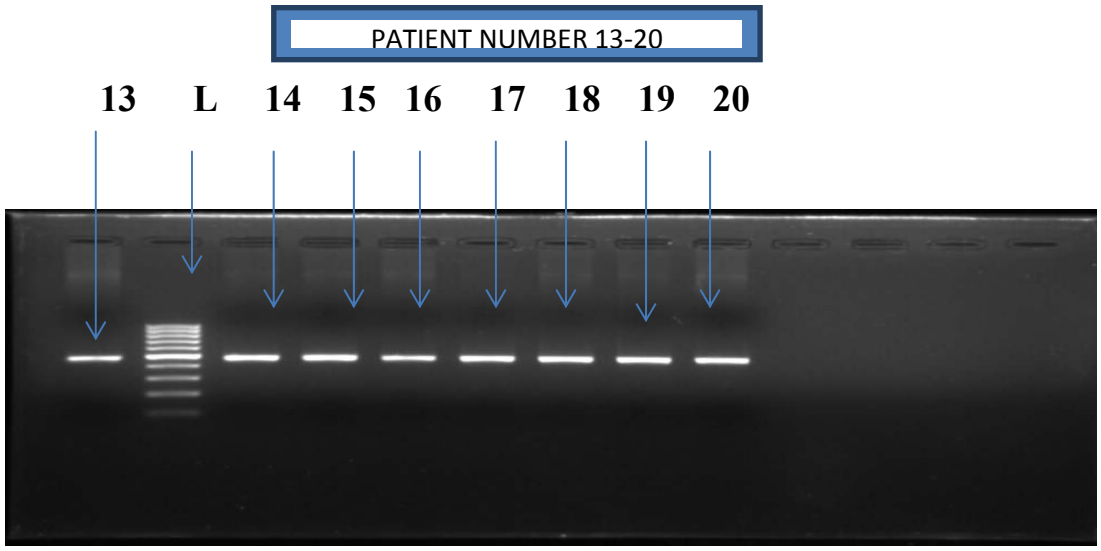
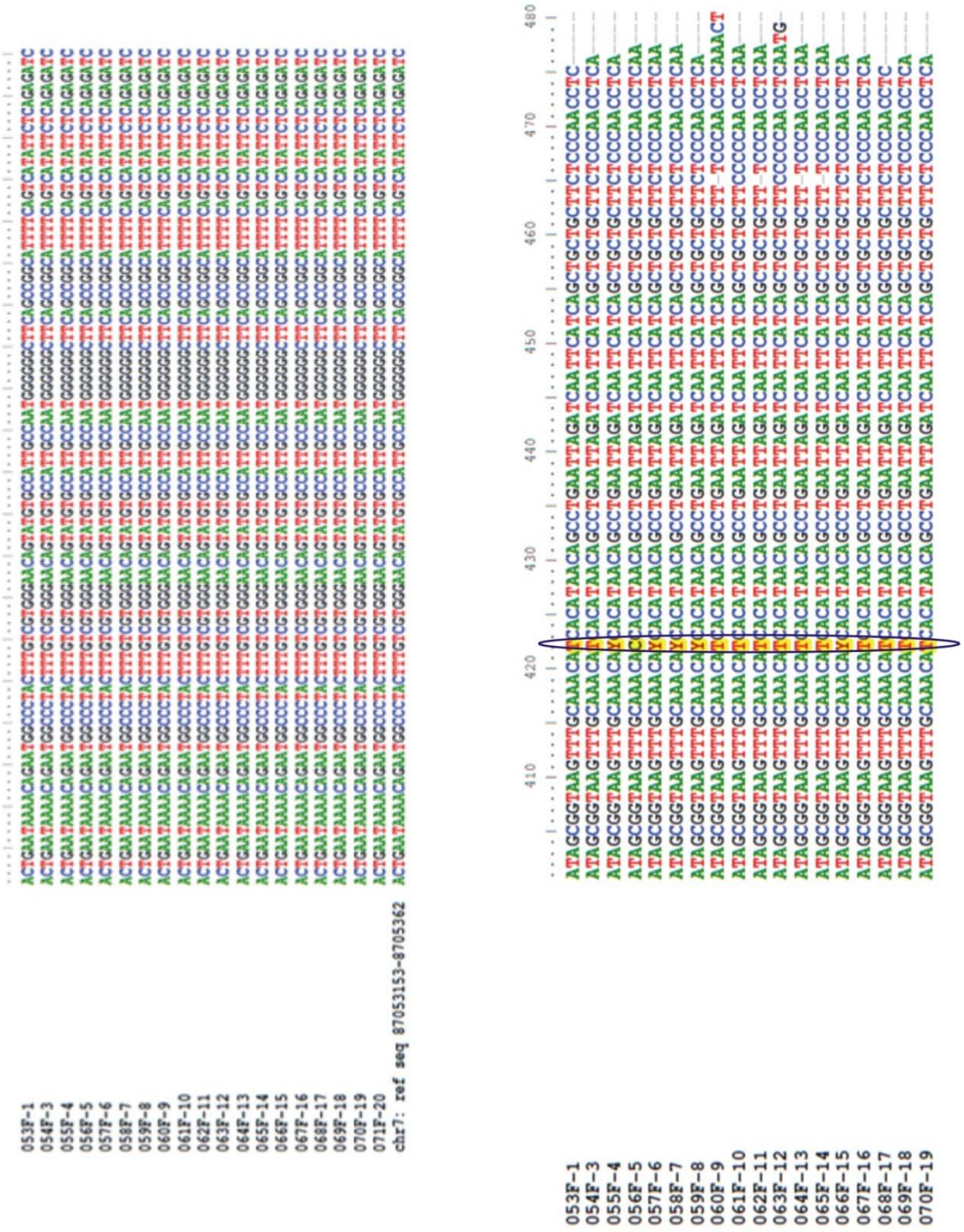


Fig: 2 PCR Amplicons of ABCB4 gene exon 17 were electrophoresed on 2% Agarose gel.

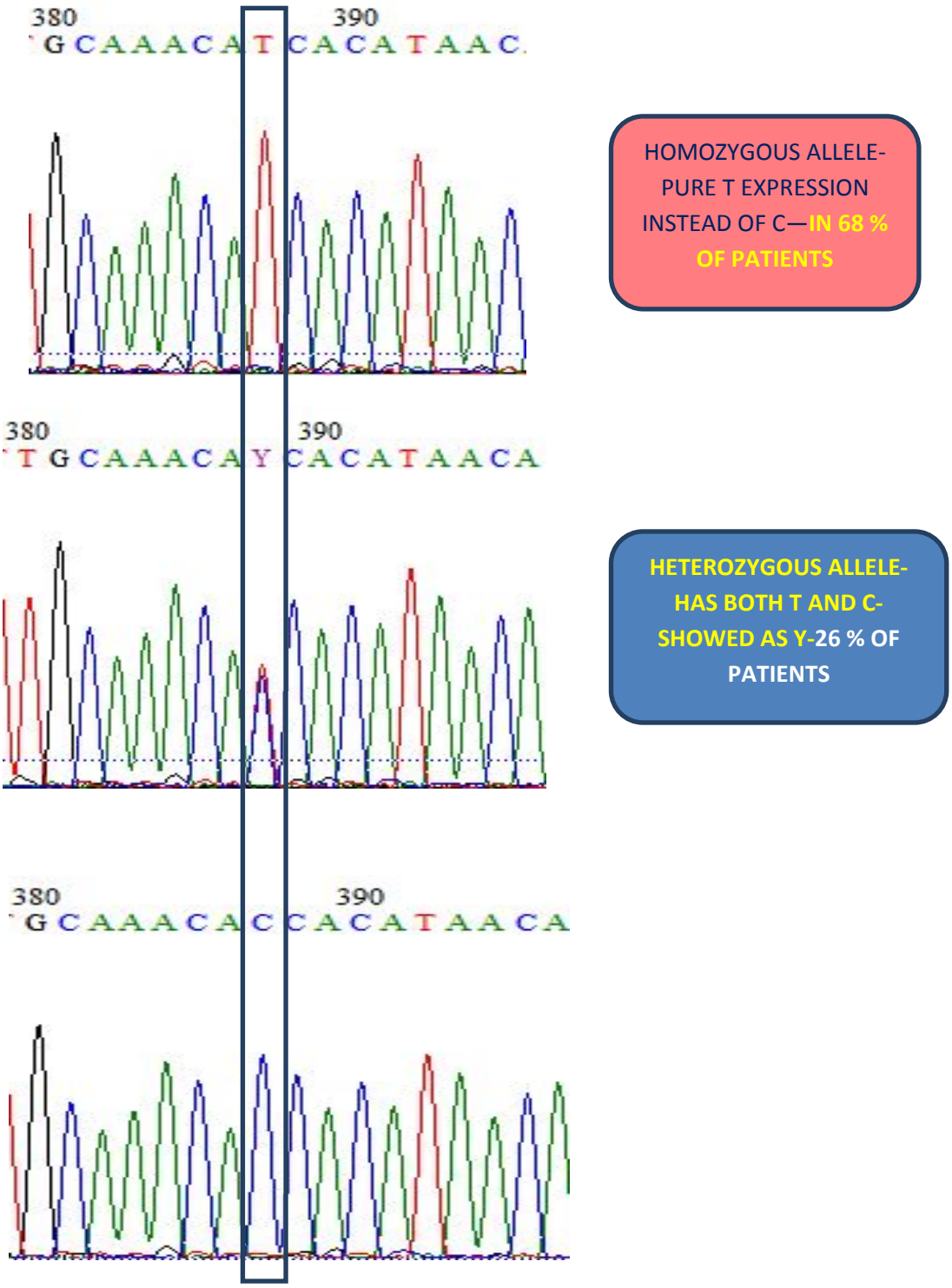
STEP NO: 3 - Matching With Primer Exon 17 of ABCB4 Gene

```
EMBOSS_001         1 GAGGCCAGAATAGGGACGGGCtaagcatcccatgaaggttatttcttggc     50
EMBOSS_001         1 --------------------------------------------------       0
EMBOSS_001        51 cgaacaacccatactcagcttatgatgtgtaatcagtaaacattgttaac    100
EMBOSS_001         1 --------------------------------------------------       0
EMBOSS_001       101 atgttataatatactgtcctactctctagtagtttgcttgtcattctctg    150
EMBOSS_001         1 --------------------------------------------------       0
EMBOSS_001       151 cacctagtttgcgagtcccttgagggcaatggccatgccttttctatgtc    200
EMBOSS_001         1 --------------------------------------------------       0
EMBOSS_001       201 tacagactctggtaactgttgtgtcatcaccagtttgccctgatgtttat    250
EMBOSS_001         1 --------------------------------------------------       0
EMBOSS_001       251 atgttctaggaagcaaatgtgccaccagtgtcctttctgaaggtcctgaa    300
                         |||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 ---------gaagcaaatgtgccaccagtgtcctttctgaaggtcctgaa     41
EMBOSS_001       301 actgaataaaacagaatggccctactttgtcgtgggaacagtatgtgcca    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        42 actgaataaaacagaatggccctactttgtcgtgggaacagtatgtgcca     91
EMBOSS_001       351 ttgccaatgggggggcttcagccggcattttcagtcatattctcagagatc    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        92 ttgccaatgggggggcttcagccggcattttcagtcatattctcagagatc141
EMBOSS_001       401 atagcggtaagtttgcaaacaccacataacagcctgaattagatcaattc    450
                     ||||||
EMBOSS_001       142 atagcg------------------------------------------    147
EMBOSS_001       451 atcaGCTGCTGCTTCTCCCAACCTC    475
EMBOSS_001       148 -------------------------    147
```

DNA SEQUENCING OF 19 PATEINTS



INTRON 17 VARIANT--DIAGNOSED BY THYMIDINE REPLACED THE CYTOSINE AT 388-INTRON 17 OF CHROMOSOME NO 7 OF ABCB4 GENE



DISCUSSION

Biliary excretion of bile salts and other bile constituents from liver cells are mediated by the canalicular (apical) transporters P-glycoprotein 3 (MDR3, ABCB4). So in this study we attempted to establish genetic variability in ABCB4 and Cholestasis. There are few published studies done in western population in which mutation and variation in ABCB4 gene has been studied

Degiorgio et al., screened mutations in 68 PFIC3 cases and found 31 mutations out of which 25 mutations were novel. The result of the study showed that the causative mutations were spread along 14 out of the 27 coding exons. But prevalence was more common on exon 17. So we studied exon 17 of ABCB4 region for genetic variation.

Low phospholipid associated Cholelithiasis is characterized by the association of ABCB4 mutations and low biliary phospholipid concentration [21].

The primary function of biliary phospholipid excretion is to protect the membranes of cells facing the biliary tree against these bile salts. The uptake of Phosphadityl choline in bile salt micelles reduces the detergent activity of these micelles[14].

The ABCB4 gene helps the efflux of phospholipid from hepatocyte into biliary canaliculi. When there is defect in function of ABCB4, it leads to production of bile low in the phospholipid which leads to increased lithogenicity[21]. ABCB4 is predominantly expressed in the liver and in small amount present in adrenal gland, muscle, testis, eye and placenta

Defect in ABCB4 has been demonstrated initially in mice[13]. The wild type mice excrete more phospholipid as compared with mice with disrupted gene, which do not excrete phospholipid [13].

De Vree et al demonstrated that there is a generalized bile acid reduction when function of ABCB4 is decreased in humans[16].

Olivier Rosmorduc et al[21] published a study in which he analyzed 60 consecutive patients with symptomatic or complicated Cholelithiasis, ABCB 4 gene mutation analysis was done in 32 patients meeting the following criteria - Age less than 40 years, recurrent symptoms after cholecystectomy-the clinical phenotype of low phospholipid associated Cholelithiasis, and 28 patients post cholecystectomy status [not meeting the criteria], 32 patients with chronic liver disease of varying etiology were taken as control. 18 patients presented with point mutation at ABCB4 locus,(in suspected clinical phenotype patients) and no mutation was seen in the other two groups.

Multivariate analysis done in these patients revealed that following factors predict the mutation.

1.Age less than 40 years

2.Recurrence of symptoms after cholecystectomy

3.Intra hepatic hyper echoic foci

In our study, we studied in 20 patients with with age less than 40 years and symptomatic Cholelithiasis after cholecystectomy

The male: female ratio is 1:4 in our study. Genomic DNA was isolated as per the procedure given and qualitative and quantitative analyses were done. Genomic DNA was electrophoresed in 0.7% agarose gel (Fig. 1). With the primer designed particularly to amplify exon 17 of ABCB4 gene, PCR product of 475bp was amplified and confirmed in 2% agarose gel (Fig. 2) the amplified PCR product was out sourced for sequencing using forward primer. The sequence result was analyzed using Bio edit software and multiple alignment of sequence all patient along with the reference sequence (NG_007118.1) from the NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NG_007118.1) is shown in result. No variations were observed in the exon 17 region of ABCB4 gene in the

patients studied.(Fig. 3) An intronic variation in the region g.51576C>T in intron 17 was observed. (Fig. 4, 5)

It is single nucleotide polymorphism at intron 17-68 % homozygous expression, 26 % -heterozygous expression, and absent in 5 %. As this variation is in intron region it does not directly affect the ABCB4 function. This is a protein –altering variants. This is predicted to have functional consequences like changes in the proteins and indirectly may leads to significant decrease in activity of ABCB4 gene.

This finding is correlated with Thomas Lang et[22] al study, who demonstrated similar mutation during the study of polymorphism of ABCB4 gene. This was a multi-centric study, and he did sequencing of 159 DNA samples of Caucasian, African-American, Japanese, and Korean origin. He identified 76 polymorphism, among them 14 exonic polymorphism, 8 protein altering variant. They reported this variation g.51576C>T in intron 17 to be in 82.7%.

On comparison we found that the mutation seen in our patients is similar to that of variant 37 mutation of Lang et al study.

The clinical significance of these studies is that when this intronic mutation is present, the patient has recurrent biliary symptoms. These patients should be placed on lifelong Urso deoxy cholic acid to prevent

complications of cholestasis as UDCA renders bile composition less injurious. This protects the hepatocytes and the biliary epithelia[24]. UDCA also enhances ABCB4 protein levels 25, suggesting one another mechanism by which UDCA may benefits ABCB4 mutation carriers[24]

This is the first Indian study to study the prevalence of ABCB4 gene mutations in patients with recurrent biliary symptoms. Larger studies are required to look into the prevalence of this mutation in our population, both in the healthy as well as those with disease.

In young patients with recurrent biliary symptoms after cholecystectomy, possible underlying genetic factors should be evaluated. These patients can benefit from medical management by Urso deoxy cholic acid therapy.

CONCLUSION

This is first study to screen variations in ABCB4 gene in young patients with gall stones in India. Mutation can be spread along entire length of the gene. We could not find any mutation in Exon 17 of ABCB4. Gene.An intronic variation in g.51576C>T in intron 17 was observed in our patients.

This implies that this mutation can have functional defects in the action of proteins leading to lithogenic bile, larger studies covering all the exons of ABCB4 gene might throw more light in genetics of this transporter defects in Indian Population.

If a mutation can demonstrated in these patients might benefitnfrom lifelong UDCA therapy decrease the lithogenicity of bile.

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ABC	-ATP-binding cassette
AE2	chloride-bicarbonate anion exchanger isoform 2
ASBT	apical Na^+ bile acid transporter
BSEP	bile salt export pump
CFTR	cystic fibrosis trans membrane regulator
FIC1	P-type ATPase mutated in progressive familial intrahepatic cholestasis type 1
MDR	multidrug resistance protein
MRP	multidrug resistance–associated protein
NPC1L1	Niemann-Pick C1 Like 1
NTCP	Na^+-taurocholate cotransporting polypeptide
OST	organic solute transporter
OATP	organic anion transporting polypeptide
SLC	solute carrier

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No : 044 25305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To
Dr.Radha M
PG in DM (MGE)
Department of Medical Gastroenterology,
Madras Medical College,Chennai -3

Dear Dr.Radha M,

The Institutional Ethics committee of Madras Medical College, reviewed and discussed your application for approval of the proposal entitled "Prevalence of mutations in MDR3/ABCB4 gene in young patients with Choleithiassis"No.35112012.

The following members of Ethics Committee were present in the meeting held on 01.11.2012 conducted at Madras Medical College, Chennai -3.

1. Prof. R. Nandhini MD -- Member Secretary
   Director, Instt. of Pharmacology ,MMC, Ch-3
2. Prof. Reghu MD -- Member
   Director , Inst. Of Internal Medicine, MMC, Ch-3
3. Prof. Shyamraj MD -- Member
   Director i/c , Instt. of Biochemistry , MMC, Ch-3
4. Prof. P. Karkuzhali. MD -- Member
   Prof., Instt. of Pathology, MMC, Ch-3
5. Prof. G.Muralidharan MS -- Member
   Prof of Surgery, MMC, Ch-3
6. Thiru. S. Govindsamy. BA,BL -- Lawyer

We approve the proposal to be conducted in its presented form.

Sd/ Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, and SAE occurring in the course of the study, any changes in the protocol and patients information / informed consent and asks to be provided a copy of the final report.

R Nandhini

Member Secretary, Ethics Committee

MASTER CHART

NAME	AGE	SEX	DIAGNOSIS	OGD	CBC	FBS	UREA	CREAT	TB	PT/INR	OTHER
JULIET	17	f	CALCULOUS CHOLECY	NAD	7600/63/31/5	78	21	0.8	1.2	1.06	CBD STONE-ERCP DONE
GUNA SUNDARI	17	f	CALCULOUS CHOLECYSTITIS		6300/61/36	109	18	0.7	0.8	1.01	NIL
PADMAVATHY	18	f	CALCULOUS CHOLECYSTITIS		8900/66/32	98	17	0.8	1.1	1.01	CBD STONE
KUMARI	18	f	CALCULOUS CHOLECYSTITIS		9100/64/32/3	89	24	0.9	1	1.04	NIL
GEETHA DEVI	21	f	CALCULOUS CHOLECYSTITIS		6400/61/30/1	78	23	0.8	0.8	1.06	ASTHMATIC
RAVI KUMAR	23	m	CALCULOUS CHOLECYSTITIS		7500/60/36	66	22	0.7	0.7	1.01	CBD STONE
RASIYA	23	f	CALCULOUS CHOLECYSTITIS		8700/63/32	80	21	0.8	0.9	1.01	
INDIRA	24	f	CALCULOUS CHOLECYSTITIS		9800/66/35	78	18	0.9	0.8	1.04	
VENKARARAMAIYA	24	m	CALCULOUS CHOLECYSTITIS		7800/68/34	109	17	1	1.1	1.1	
ANJALIDEVI	25	f	CALCULOUS CHOLECYSTITIS		8900/67/37	98	24	1.1	1.2	1.1	
BAGYALAKSHMI	26	f	CALCULOUS CHOLECYSTITIS		9500/64/33	98	23	0.8	1.2	1.1	
VIDAVAREIYAR	26	m	CALCULOUS CHOLECYSTITIS		8900/62/34/3	89	22	0.7	1.3	1.1	
MURUGANMMAL	26	f	CALCULOUS CHOLECYSTITIS		9100/64/32	78	24	0.8	1.1	1.06	
JANSI RANI	27	F	CALCULOUS CHOLECY	FUNDAL G/	9800/52/37/10.6/B	89	34	0.9	0.8	1.07	AUDIOGRAM -HIGH FREQ HEARING LOSS
LAXMI NARAYANMMAL	27	f	CALCULOUS CHOLECYSTITIS		9500/64/33	88	22	1	0.9	1	CLD
ALI FATHIMA	28	f	CALCULOUS CHOLECY	LAX LES	79OO/64/31	102	28	0.8	1.2	1.02	ASTHMATIC
HYARUNISHA	28	f	CALCULOUS CHOLECYSTITIS		7500/60/36	98	24	0.7	0.9	1.06	
MAJITHA BANU	31	f	CALCULOUS CHOLECYSTITIS		8700/63/32	110	23	0.8	0.8	1.01	
TER CHART	24	f	CALCULOUS CHOLECYSTITIS		9800/66/35	89	22	0.9	1.1	1.01	
RANI	26	f	CALCULOUS CHOLECYSTITIS		7800/68/34	78	21	0.8	1.2	1.04	

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Dissertation submitted for

D.M.DEGREE EXAMINATION- AUGUST-2013

BRANCH-IV-MEDICAL GASTROENTEROLOGY

MADRAS MEDICAL COLLEGE

&

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Paper ID	311829772
Paper title	PREVALENCE OF MUTATIONS IN MDR3/ABCB4 GENE IN YOUNG PATIENTS WITH CHOLELITHIASIS D
Assignment title	Medical
Author	M.radha 16102504 D.M. Medical Gastroenterology
E-mail	drradha0509@gmail.com
Submission time	23-Mar-2013 02:20PM
Total words	6144

First 100 words of your submission

PREVALENCE OF MUTATIONS IN MDR3/ABCB4 GENE IN YOUNG PATIENTS WITH CHOLELITHIASIS Dissertation submitted for D.M.DEGREE EXAMINATION- AUGUST-2013 BRANCH-IV-MEDICAL GASTROENTEROLOGY MADRAS MEDICAL COLLEGE & RAJIV GANDHI GOVERNMENT GENERAL HOSPITAL CHENNAI-600003 THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY CHENNAI-600032 Introduction Bile is secreted from the hepatocyte and it is essential for lipid metabolism, excretion of xenobiotic, and cholesterol homeostasis. Bile secretion depends upon the formation of bile acids in hepatocyte and its canalicular secretion. Once secreted in to the bile duct, bile enters the small bowel through the ampulla of water during the digestion phase. Bile stored...